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14. ABSTRACT

Skeletal muscle injuries, caused by intense exercise or trauma, are among the most common injuries in military personnel. Enhancement of muscle repair following injury would minimize time lost and maximize performance during training and combat. We and others have published data demonstrating that the extracellular protease urokinase-type plasminogen activator (uPA) is required for efficient muscle repair, although the underlying mechanisms remain to be elucidated. In the present project, immunofluorescence analysis demonstrated that cell proliferation is accelerated in injured muscle of PAI-1 null mice compared to wild-type mice. Immunofluorescence analysis also demonstrated that satellite cell accumulation is increased in injured muscle of PAI-1 null mice compared to wild-type mice. Western blot analysis supports accelerated cell proliferation and satellite cell accumulation in injured muscle of PAI-1 null mice. Taken together, these data indicate that satellite cell activity is enhanced in injured muscle of PAI-1 null mice. Findings from continued work on this project will provide insight into potential manipulation of components of the plasminogen system as a way to enhance muscle repair. Enhancing muscle repair following injury would minimize time lost due to muscle injury both during training and combat, and maximize performance following return from injury.

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Skeletal muscle repair, muscle function, urokinase-type plasminogen activator, satellite cell, hepatocyte growth factor

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Table of Contents

Introduction

Proper skeletal muscle function is essential for nearly all activities required for military training and combat. Injury to skeletal muscle caused by intense exercise or trauma compromises muscle function, and such injuries are among the most common experienced by military personnel. Enhancing muscle repair following injury would minimize time lost due to muscle injury both during training and combat, and maximize performance following return from injury. Our published data indicates that the extracellular protease urokinase-type plasminogen activator (uPA) is required for efficient muscle repair [1], although the underlying mechanisms remain to be elucidated. One way that uPA could promote repair is by stimulating activity of satellite cells; satellite cells are muscle stem cells required for repair. One mechanism by which uPA could stimulate satellite cell activity is by activating hepatocyte growth factor (HGF); HGF can activate quiescent satellite cells, and stimulate their proliferation and migration. *The guiding hypothesis of this proposal is that the balance of uPA and its endogenous inhibitor, PAI-1, regulates muscle repair.* The purpose of the present project is to determine whether the balance of uPA and PAI-1 regulates activation of HGF and activation and proliferation satellite cells during muscle repair.

Body

Task 1. In the Statement of Work for this project, Task 1 is to determine whether the balance of uPA and PAI-1 regulates satellite cell activation and proliferation following muscle injury (Months 1-18 of the project). For Task 1a and 1c, wild-type and PAI- null mice were obtained from Jackson Laboratories, and breeding colonies established. The extensor digitorum longus (EDL) muscle of wild-type mice and PAI-1 null mice was injured using injection of snake toxin (cardiotoxin), and muscles harvested at 0.5, 1, 3, 5 and 10 days post-injury. The right EDL muscle was processed for frozen sections and the left EDL muscle was processed for Western blotting. Bromodeoxyuridine (BrdU) was injected into mice 1 hour before euthanasia; BrdU is incorporated into newly synthesized DNA and immunofluorescent detection of BrdU in muscle sections was used as a marker for proliferating cells. Satellite cells were detected by immunolabeling of cryosections for the myogenic transcription factor MyoD. Cells positive for MyoD or BrdU were counted on two sections per muscle and averaged across muscles.

Very few BrdU positive cells were observed in control muscle or at 0.5 or 1 day post-injury in wild-type and PAI-1 null mice. For wild-type mice, BrdU positive cells increased at 3 days post-injury, peaked at 5 days post-injury and then decreased again at 10 days post-injury (Figure 1). For PAI-1 null mice, BrdU positive cells peaked at 3

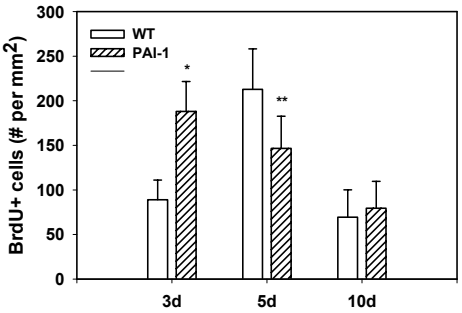

Figure 1. Cell proliferation is accelerated in injured muscle of PAI-1 null mice compared with wild-type mice. Values shown are means with standard error bars. * value for PAI-1 null mice significantly greater than that for wild-type (WT) mice. ** value for PAI-1 null mice significantly smaller than that for wild-type mice. N = 8, $p < 0.05$.

days post-injury, with a progress decline thereafter. Analysis of variance indicated that differences between wild-type and PAI-1 null mice were significant at 3 and 5 days post-injury ($p < 0.05$). These data indicate significantly accelerated cell proliferation in PAI-1 null mice compared with wild-type mice, consistent with the accelerated healing observed in a previous study [1].

MyoD staining has been performed in cross-sections of muscle from wild-type and PAI-1 null mice harvested at 3 and 5 days post-injury. At 3 days post-injury, analysis of variance indicated that the number of MyoD positive cells was significantly greater in muscle of PAI-1 null mice compared with wild-type mice ($p < 0.05$, Figure 2). Since all of these MyoD positive cells were small mononuclear cells, these data indicate significantly enhanced accumulation of satellite cells at this time point. At 5 days post-injury, the number of MyoD positive cells was still significantly larger in muscle of PAI-1 null mice compared with wild-type mice. Many of these MyoD positive cells showed morphology of regenerating muscle fibers, indicating that satellite cells had begun to fuse at this time point. MyoD positive cells have been extremely difficult to identify at 0.5 and 1 day post-injury because of background staining. Many different avenues have been attempted to reduce this background staining, including different primary and secondary antibodies, different concentrations of antibodies, and different block reagents. None of these attempts have been successful to date. However, the data obtained strongly indicate that satellite cell proliferation and accumulation is increased in PAI-1 null mice compared with wild-type mice.

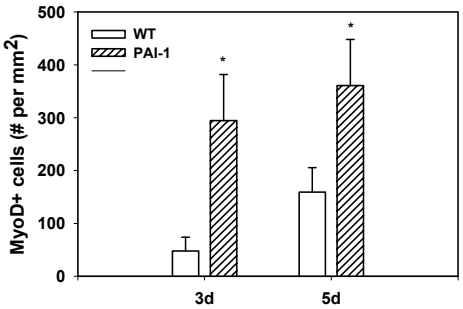

Figure 2. Satellite cell activity is increased in injured muscle of PAI-1 null mice compared with wild-type mice. Values shown are means with standard error bars. * value for PAI-1 null mice significantly greater than that for wild-type (WT) mice. N = 8, $p < 0.05$.

Western blots of muscle homogenates using antibodies against proliferating cell nuclear antigen (PCNA) and MyoD were used to corroborate data obtained from immunostained sections. For wild-type mice, PCNA demonstrated a progressive increase to 5 days post-injury, with a decline at 10 days (Figure 3). MyoD showed a similar trend as PCNA, apparently peaking at 5 days (Figure 4). For PAI-1 null mice, PCNA showed a strong increase to 3 days post-injury, with a progress decline to 10 days. Again, MyoD showed a similar trend as PCNA, apparently peaking at 3 days.

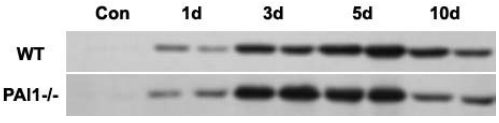

Figure 3. Western blot of PCNA using muscle homogenates of injured muscle of wild-type and PAI-1 null mice. Note that there is an indication of an earlier peak of PCNA levels in PAI-1 null compared with wild-type mice.

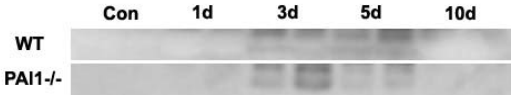

Figure 4. Western blot of MyoD using muscle homogenates of injured muscle of wild-type and PAI-1 null mice. Note that there is an indication of an earlier peak of MyoD levels in PAI-1 null compared with wild-type mice.

Further Western blots will be performed with additional samples from wild-type and PAI-1 null mice to assess the protein levels of PCNA and MyoD, then blots will be analyzed using densitometry, and the resulting data compared using analysis of variance. The Western blot data so far collected support the immunofluorescence data, further indicating that satellite cell proliferation and accumulation is increased in PAI-1 null mice compared with wild-type mice.

The number of satellite cells in uninjured muscle will also be counted to determine whether the number of quiescent cells differs between wild-type and PAI-1 null mice. Cross sections from uninjured muscles will be labeled with a monoclonal antibody against the satellite cell marker Pax7 to identify quiescent satellite cells. To date, the number of Pax7 positive cells has been counted in two sections per muscle for 6 wild-type and 2 PAI-1 null muscles. These preliminary data indicate that the number of satellite cells in uninjured muscle of wild-type mice (15.3 $\pm$ 3.9, n = 6) is similar to the number in uninjured muscle of PAI-1 null mice (12.4 $\pm$ 4.5; n = 2).

For Task 1b, EDL muscles of uPA null mice will be injured to determine whether loss of uPA results in impaired satellite cell activity. uPA null mice are not efficient breeders, and this has caused a delay both in shipment of mice from Jackson Laboratories, and establishment of a breeding colony. The breeding colony is now established, and we are currently performing the first injuries with the uPA null mice. We expect that Task 1b will be completed on time, by month 18 of the project.

Task 2. Task 2 is to determine whether the balance of uPA and PAI-1 regulates satellite cell migration and fusion (Months 13-30 of the project). We have started to establish procedures in our laboratory for isolating satellite cells from neonatal mouse muscle. Muscle has been harvested, finely minced, and digested using pronase. After trituration to dissociate cells from tissue debris, the suspension has been filtered, centrifuged and the satellite cells isolated on a Percoll gradient. Cells were then plated in F-10 media supplemented with 20% fetal bovine serum, 2.5 ng/ml basic fibroblast growth factor, and 1% penicillin/streptomycin. Our preliminary experiments with this technique have produced a substantial yield of satellite cells. However, we have had difficulties with fibroblast contamination of the cultures and are currently testing procedures to minimize fibroblasts (preplating steps, light trypsinization steps). Once we optimize this procedure, we will then move on to Task 2a, to determine satellite cell migration and fusion in cells isolated from wild-type, uPA null and PAI-1 null mice.

Key Research Accomplishments

- Established breeding colonies of wild-type, uPA null and PAI-1 null mice
- Immunofluorescence analysis demonstrated that cell proliferation is accelerated in injured muscle of PAI-1 null mice compared to wild-type mice
- Immunofluorescence analysis also demonstrated that satellite cell accumulation is increased in injured muscle of PAI-1 null mice compared to wild-type mice
- Preliminary Western blot analysis supports accelerated cell proliferation and satellite cell activity in injured muscle of PAI-1 null mice compared with wild-type mice
- Preliminary immunohistochemical analysis indicates no difference in number of quiescent satellite cells in uninjured muscle of wild-type and PAI-1 null mice
- Satellite cell isolation procedure is under development

Reportable Outcomes

None to date. We anticipate presenting preliminary data at the 2006 Experimental Biology meeting in San Francisco, and at the 2006 DOD Military Health Research Forum in San Juan. We also anticipate preparing a manuscript for publication at the end of 2006.

Conclusion

Our experiments to date indicate that cell proliferation and satellite cell accumulation are enhanced in injured muscle of PAI-1 null compared to wild-type mice. These findings are consistent with the accelerated repair and functional recovery we observed in injured muscle of PAI-1 null compared to wild-type mice [1]. In year 2 of the grant, we will finish Task 1 and determine whether loss of uPA impairs satellite cell activity following injury. We will also begin work on Task 2 to determine whether the balance of uPA and PAI-1 regulate satellite cell migration and fusion in vitro and in vivo. Findings from this work will provide insight into potential manipulation of components of the plasminogen system as a way to enhance muscle repair. Enhancing muscle repair following injury would minimize time lost due to muscle injury both during training and combat, and maximize performance following return from injury.

References

1. **Koh TJ, SC Bryer, AM Pucci, and TH Sisson**. Mice deficient in plasminogen activator inhibitor-1 have improved skeletal muscle regeneration. *Am J Physiol Cell Physiol*. 289:C217-23, 2005.